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## STATE-OF-THE-ART REVIEW

# Membrane targeting of core autophagy players during autophagosome biogenesis

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**Keywords**

ATG; autophagosome; autophagy; membrane recruitment; phagophore; PI(3)P

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Autophagosomes are vital organelles required to facilitate the lysosomal degradation of cytoplasmic cargo, thereby playing an important role in maintaining cellular homeostasis. A number of autophagy-related (ATG) protein complexes are recruited to the site of autophagosome biogenesis where they act to facilitate membrane growth and maturation. Regulated recruitment of ATG complexes to autophagosomal membranes is essential for their autophagic activities and is required to ensure the efficient engulfment of cargo destined for lysosomal degradation. In this review, we discuss our current understanding of the spatiotemporal hierarchy between ATG proteins, examining the mechanisms underlying their recruitment to membranes. A particular focus is placed on the relevance of phosphatidylinositol 3-phosphate and the extent to which the core autophagy players are reliant on this lipid for their localisation to autophagic membranes. In addition, open questions and potential future research directions regarding the membrane recruitment and displacement of ATG proteins are discussed here.

## Introduction

The engulfment of cellular cargo by autophagosomes and the eventual recruitment of lysosomes are essential steps in the clearance of unwanted material during autophagy [1]. This *de novo* biogenesis of autophagosomes requires a series of core autophagy-related (ATG) protein complexes and membrane sources. Defects in autophagosome maturation reduce their ability to recruit lysosomal hydrolases, leading to deleterious effects on cellular function and homeostasis. Increasing research has focused on understanding how the activities of these ATG proteins are regulated in

cells to mediate the formation of autophagosomes and, subsequently, cargo degradation.

Autophagosome biogenesis is catalysed by various complexes that can crosstalk in order to facilitate the efficient progression of autophagy (Fig. 1) [2]. Of these, the unc-51-like kinase (ULK) complex is subjected to post-translational modifications by upstream signalling complexes to regulate the initiation of autophagy and the vacuolar protein-sorting 34 (VPS34) complex can modify the composition of membranes. These events activate downstream effectors

**Abbreviations**

AP, adaptor protein complex; ATG, autophagy-related; BATS, Barkor/ATG14 autophagosome targeting sequence; CCD, coiled-coil domain; DFCP1, double FYVE-containing protein 1; FIP200, FAK family-interacting protein of 200 kDa; GABARAP, gamma-aminobutyric acid receptor-associated protein; HORMA, Hop1, Rev7 and Mad2; LC3, microtubule-associated protein 1A/1B-light chain 3; LIR, LC3-interacting region; MAM, mitochondrial-associated ER membrane; MLD, MAM-localisation domain; mTORC1, mammalian target of rapamycin complex 1; PE, phosphatidylethanolamine; PI(3)P, phosphatidylinositol 3-phosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(5)P, phosphatidylinositol 5-phosphate; TOM, translocase of the outer mitochondrial membrane; TRAPP, transport protein particle; ULK, unc-51-like kinase; UVRAG, UV radiation resistance-associated gene protein; VPS34, vacuolar protein-sorting 34; WIPI, WD repeat domain phosphoinositide-interacting.

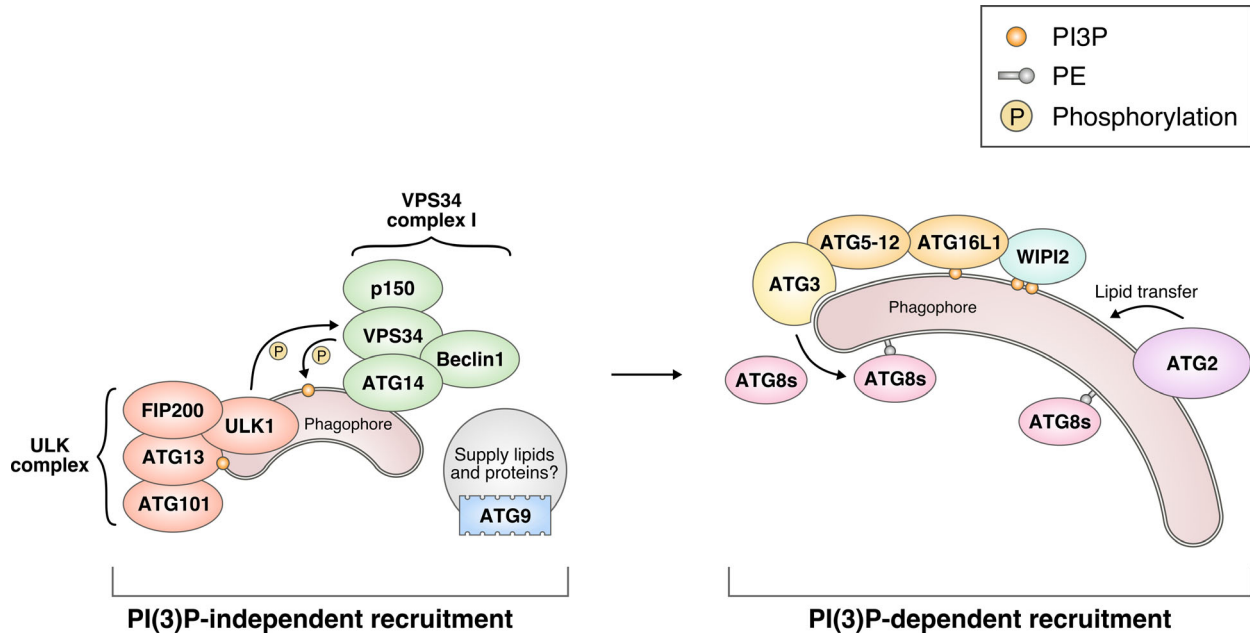
that lead to the lipid conjugation of a family of ubiquitin-like proteins, ATG8s [divided into two subfamilies: microtubule-associated protein 1A/1B-light chain 3 (LC3) and gamma-aminobutyric acid receptor-associated protein (GABARAP)]. The lipidation of ATG8s is catalysed by a three-step reaction involving ATG7, ATG3 and the ATG16L1-ATG5-ATG12 complex. Cargo recruitment can occur at various stages of autophagosome biogenesis, and multiple lipid sources may contribute to autophagosome growth [3].

Autophagy is a highly conserved pathway across eukaryotes. The identification of ATG genes (Atg1-Atg15) was first performed in yeast [4], and their mammalian homologues have since been identified. A similarity in the spatiotemporal hierarchy between autophagy proteins is also observed between the yeast and mammalian systems [5,6]. This involves the initial activation of the ULK/Atg1 (denoting mammalian/yeast proteins) and VPS34/Vps34 complexes and ATG9/Atg9. These players facilitate the recruitment of further downstream machinery including the WD repeat domain phosphoinositide-interacting proteins (WIPI/Atg18 and Atg21), the ATG5/Atg5 complex and ATG2/Atg2, resulting in the lipid conjugation of the ATG8/Atg8 family of proteins.

Membrane targeting of most ATG proteins to the pre-autophagosomal structure, herein referred to as the phagophore, is essential for their activities during autophagy. Formation of 'punctate' structures that are positive for one or more ATG proteins is the commonly accepted readout to determine protein targeting to autophagic membranes. How, when and what regulates the recruitment and displacement of autophagy players at the phagophore are ongoing questions. Although extensive research has been performed in various model organisms to identify the proteins and lipids involved in autophagy, we will focus here on discussing our current understanding of how ATG proteins are recruited to autophagosomal membranes in mammalian cells and explore unanswered questions to be addressed in future research. Where research using mammalian systems is lacking, we will discuss potential mechanisms of membrane targeting based on studies in yeast [7].

## Regulation of autophagy by lipid signatures

Autophagosome biogenesis involves a series of changes in membrane lipid composition [3,8]. Lipids can influence membrane charge, structure, fluidity and



**Fig. 1.** An overview of autophagosome biogenesis. Following nutrient starvation, the ULK complex (through ATG13 and FIP200), the VPS34 complex I (through ATG14) and ATG9-containing vesicles translocate to the phagophore in the absence of PI(3)P synthesis. The ULK1 kinase can phosphorylate and activate the VPS34 complex I, triggering the generation of PI(3)P on the phagophore. This results in the recruitment of further downstream players, such as WIPI2, the ATG5 complex and ATG2. The recruitment of the ATG5-ATG12 conjugate through binding to ATG16L1 triggers the conjugation of ATG8s to PE. The eventual maturation of the autophagosome leads to the displacement of most ATG proteins and its fusion with lysosomes (not shown).

curvature, thus mediating the recruitment of effector proteins and enzymatic reactions involving the lipid conjugation of ATG8 proteins [9,10]. Various lipids have been shown to influence autophagosome biogenesis, and the effector proteins and mechanisms involved are topics of ongoing research [8]. Phosphoinositides are one subclass of phospholipids, and their roles in autophagy have been studied extensively [8]. Their phosphorylation variants, including phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P), phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>) and phosphatidylinositol 3,5-bisphosphate (PI(3,5)P<sub>2</sub>), are present on various cellular membranes where they regulate signalling and trafficking events [11]. Of these variants, PI(3)P plays an essential role in initiating the recruitment of several ATG proteins to the phagophore. Furthermore, by examining the localisation of a PI(3)P-binding protein called double FYVE-containing protein 1 (DFCP1), it has been shown that phagophores emanate from PI(3)P-enriched sites on the ER termed omegasomes [12,13]. However, the specific function of DFCP1 during omegasome formation remains unknown as its knockdown does not compromise autophagic activity in cells [12].

The synthesis of PI(3)P is mainly catalysed by an autophagy-specific complex, the lipid kinase VPS34 complex I. However, other lipid kinases and phosphatases have been shown to contribute to cellular PI(3)P availability [14]. Given that PI(3)P can also be found on nonautophagic membranes [15], it is likely to be necessary but not sufficient for the membrane recruitment of ATG proteins. Other phosphoinositide variants also appear to be important during autophagy. For instance, it has been shown that PI(5)P can compensate for prolonged PI(3)P depletion during autophagy [16]. In addition, PI(4)P can influence the localisation of ATG proteins to the phagophore [17]. In this review, we will focus on the relevance of PI(3)P and discuss how ATG proteins are recruited to autophagosome membranes by dividing these into two categories: PI(3)P-independent and PI(3)P-dependent recruitment.

## PI(3)P-independent recruitment

### ULK complex

The ULK complex (composed of ULK1/2, ATG13, FAK family-interacting protein of 200 kDa (FIP200) and ATG101) can be regulated by upstream signalling complexes, such as mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein

kinase [18]. Upon activation, the ULK complex can relay cues to downstream autophagy players through protein–protein interactions and phosphorylation events. ULK complex activation represents one of the earliest signals during autophagosome biogenesis, although involvement of the complex in further downstream stages has also been reported [18]. Whilst the initial localisation of the ULK complex to the phagophore is thought to occur independently of VPS34 activity [19], PI(3)P was shown to stabilise it on the phagophore by directly interacting with ATG13 [13].

The relevance of the recruitment of the ULK complex to the phagophore and whether it is required to initiate the phosphorylation of its targets remain unclear. Unlike the mammalian ULK complex, which is constitutively assembled, the assembly of the yeast Atg1 complex (homologous to the mammalian ULK complex) is induced upon autophagy activation by TorC1 inhibition [20–23]. The dissimilarity between the binding of mammalian ULK1 and yeast Atg1 proteins to their complex members (constitutive versus induced) may reflect a mechanism tailored to accommodate a more complex autophagy activation in higher organisms that can occur in the absence of mTORC1 inactivation and/or during cargo selection.

Genetic inhibition of ATG13 or FIP200 impedes the localisation of ULK1 to the phagophore [19–20,24]. Similarly, mutants of ATG13 that cannot bind ULK1 or FIP200 also inhibit the puncta formation of these proteins [25,26]. The loss of ULK1 recruitment to the phagophore in the absence of ATG13 or FIP200 binding could be a result of its inhibited kinase activity, which requires the assembly of the ULK complex [20,27]. In line with this, it has been shown that a kinase-dead ULK1 mutant did not localise to the phagophore and was deficient in inducing ATG16L1 puncta formation [24,28]. Furthermore, genetic inhibition of ATG13 or FIP200 in cells was shown to destabilise the ULK complex, which may also explain why losing any of these components prevents their detection on the phagophore [24,29,30].

Mechanistically, aside from its interactions with the other ULK complex members, the ability of ATG13 to interact with acidic phospholipids, including PA, PI(3)P and PI(4)P, has been shown to be important for its recruitment to punctate structures [13]. However, mutants of ATG13 that inhibited its membrane targeting did not perturb LC3 lipidation induced by amino acid starvation, suggesting that ATG13 puncta formation, in some cases, may not be essential to trigger downstream events [25,26]. These findings could be due to the induction of noncanonical ULK-independent autophagy, an alternative role of the ULK

complex in downstream stages, or simply adaptation of cells to prolonged knockout of ATG proteins and therefore in need of more careful investigations.

The contribution of ATG101 appears to be different from ATG13 and FIP200. ATG101 knockout did not impede FIP200 puncta formation, although these puncta were present even in nutrient-rich conditions [31]. Perturbing the interaction between ATG101 and ATG13 disrupted the recruitment of ATG101 to the phagophore and subsequently inhibited GFP-LC3 lipidation. Structurally, ATG101 is thought to consist of a Hop1, Rev7 and Mad2 (HORMA) domains, through which it can dimerise with the N-terminal HORMA domain on ATG13 [31,32]. Binding to ATG13 facilitates the recruitment of ATG101 to the phagophore, which can subsequently recruit the downstream lipidation machinery in a manner dependent on conserved tryptophan and phenylalanine residues, termed the WF finger, within ATG101. This suggests that ATG101 plays a role in recruiting the downstream autophagy players potentially by stabilising and activating ATG13 [26,29,31,33]. Additionally, whilst mutating the phospholipid-binding or HORMA domain on ATG13 reduces its recruitment to punctate structures, constructs bearing mutations to both of these sites seem to be persistently recruited to punctate structures that are also positive for FIP200 even in the absence of autophagy induction [26]. These intriguing observations indicate that binding of ATG13 to phospholipids and ATG101 may be important for the proper removal of ATG13 from phagophores or for autophagosome maturation.

Components of the ULK complex also harbour the ability to interact with downstream autophagy players, including ATG16L1, members of the ATG8 family and cargo receptor proteins. For example, an ability of FIP200 to bind the cargo receptor protein p62 through a C-terminal claw-shaped domain has been reported [34]. By being directly recruited to the cargo site, the ULK complex can activate the recruitment of the downstream autophagy machinery, leading to the localised growth of the autophagosome at these specific sites. The relevance of these interactions will be discussed further below.

### VPS34 Complex I

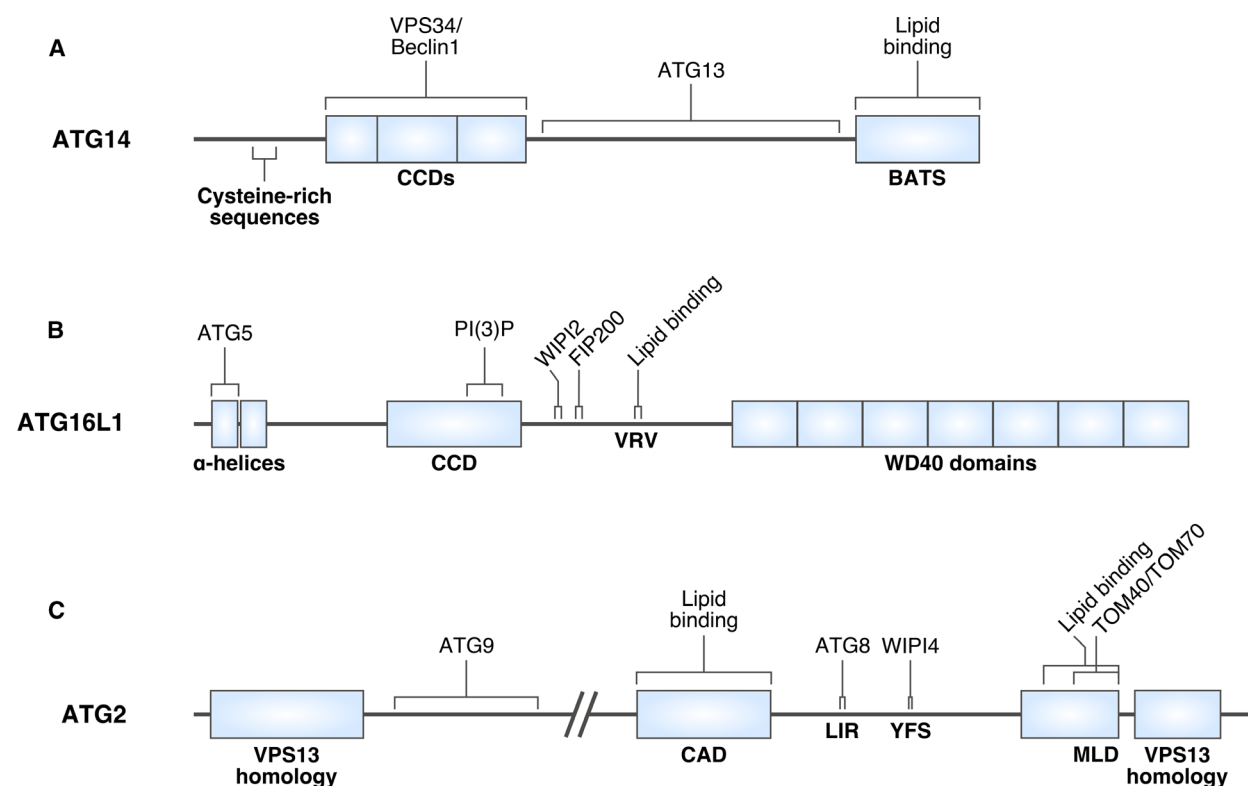
The lipid kinase VPS34 exists in two complexes, where the mutually exclusive presence of either ATG14 or UV radiation resistance-associated gene protein (UVRAG) specifies its involvement in autophagy (complex I) or endocytosis (complex II), respectively [35]. The production of PI(3)P by VPS34 is critical for autophagosome maturation and for various stages of endocytic trafficking. It is thought that the ULK

complex can phosphorylate members of the VPS34 complex I in a manner dependent on an interaction between ATG13 and ATG14 [36], thereby activating the production of PI(3)P following mTORC1 inactivation [36–38]. Chemical inhibition of VPS34 does not impede the recruitment of the complex to the phagophore, suggesting that it occurs in the absence of VPS34 lipid kinase activity [35].

The VPS34 complex I is composed of VPS34, ATG14, Beclin 1 and p150. The absence of ATG14 from VPS34 complex II implies its relevance in specifically recruiting VPS34 complex I to autophagosome initiation sites (Fig. 2A) [35,39]. Cysteine-rich sequences within the N-terminal half of ATG14 were shown to be important for its ER localisation and for autophagy [40]. Point mutants of these residues disrupted the localisation of ATG14 to the phagophore but retained binding to members of the VPS34 complex I. On the other hand, large deletions in the coiled-coil domains (CCDs) of ATG14 abrogated its interaction with VPS34 and Beclin 1 but did not affect its punctate localisation, suggesting that components of the VPS34 complex I are dispensable for the recruitment of ATG14 to the phagophore [35]. Surprisingly, this CCD deletion mutant of ATG14 was still able to colocalise with the downstream autophagy player, ATG16L1, despite the lack of VPS34 binding and, presumably, VPS34 activity at these autophagic structures. These findings suggest that other lipids or lipid kinases and/or phosphatases may play a role in compensating for the loss of VPS34-mediated PI(3)P biogenesis at the phagophore [14]. Interestingly, cells depleted of VPS34 still underwent significant LC3 lipidation but did not support autophagosome–lysosome fusion, potentially indicating that whilst the loss of VPS34 activity may be compensated for during autophagosome biogenesis, it is indispensable for certain activities of the endocytic pathway involved in lysosome fusion [41].

How ATG14 recognises autophagosome initiation sites (including omegasomes and/or ER–mitochondria contact sites) in a PI(3)P-independent manner remains an open question. It could possibly be mediated through the binding of ATG14 to the HORMA domain of ATG13 [36], which has previously been shown to be important for the recruitment of Atg14 to punctate structures in yeast [42]. It is also possible that ATG14 can recognise autophagosome initiation sites by directly interacting with lipids. The C terminus of ATG14 contains hydrophobic residues required for its colocalisation with autophagic markers, termed the Barkor/ATG14 autophagosome targeting sequence (BATS) domain [43]. This domain harbours an ability to bind liposome preparations generated from bovine brain





**Fig. 2.** Domains and binding partners of ATG14, ATG16L1 and ATG2, and their relevance during phagophore recruitment. (A) ATG14: Cysteine-rich sequences and the BATS domain were shown to be important for the localisation of ATG14 to the phagophore. ATG14 binding to ATG13 may also be important for its phagophore localisation. The CCDs of ATG14 mediate binding to VPS34 and Beclin 1 but are dispensable for its recruitment to the phagophore. (B) ATG16L1: Sequences within the middle region of ATG16L1 mediate its binding to PI(3)P, WIP2 and FIP200 and are required for its recruitment to the phagophore. The interaction of ATG16L1 with ATG5 through its N-terminal end is dispensable for its localisation to the phagophore but is essential for activating LC3 lipidation. On the other hand, the ATG16L1  $\beta$ -isoform-specific motif, VRV and residues within the WD40 domains are dispensable for canonical autophagy but are required for its membrane recruitment and LC3 lipidation on single membranes. (C) ATG2: ATG2 contributes to autophagosome maturation potentially through a lipid transfer capacity within its N-terminal Chorein/VPS13 homology region and by interacting with ATG8 proteins via an LIR motif within its C-terminal half. Homology between Chorein/VPS13 and the C-terminal of ATG2 has also been described, but these residues are dispensable for autophagy. Whilst its interaction with WIP4 via a YFS motif is thought to be dispensable for autophagy, the ability of ATG2 to interact with ATG9 (through sequences within its N-terminal half) and TOM40/TOM70 (through the MLD C-terminal region) is thought to facilitate its recruitment to the phagophore and function during autophagy. Binding of ATG2 to PI(3)P through sequences within the MLD domain has also been described, but its relevance to autophagy is yet to be determined. The CAD domain is thought to mediate binding to lipids and may facilitate recruitment of ATG2 to autophagosome initiation sites. The two forward slash lines (//) indicate additional sequences that are not depicted here (~ 900 amino acids) and are deleted in order to retain the appropriate scale of the ATG2 domains.

extracts as well as liposomes containing PI(3)P and PI(4,5)P<sub>2</sub> but not other phosphoinositides. This ability of ATG14 to directly bind lipids warrants further research to dissect potential lipid compositions that may influence its membrane binding and aid its recruitment to the phagophore prior to PI(3)P synthesis.

## ATG9

The presence of six transmembrane domains on ATG9 suggests that it is constitutively membrane-bound and that its localisation to the phagophore relies on

vesicular trafficking events [44]. Indeed, ATG9 was shown to localise to the plasma membrane as well as to various vesicles of the endocytic pathway. Upon autophagy induction, ATG9 vesicles are redistributed from the Golgi apparatus and endosomes to tubular compartments surrounding the growing autophagosomes. This redistribution requires ER–Golgi retrograde transport but not PI(3)P [45] and is mediated through the activities of UVRAG [46], adaptor protein complex 4 (AP-4) [47,48] and components of the retrograde complex through an interaction between ATG9 and TBC1 domain family member 5 [49]. In addition

to regulating ATG9 trafficking, the retrograde complex can influence autophagic degradation by controlling lysosomal activity [50]. The localisation of ATG9 to recycling endosomes has also been shown to be key for autophagy [51,52]. The redistribution of ATG9-positive vesicles from recycling endosomes to autophagosomes requires residues within its N-terminal region as well as the activities of sorting nexin 18, dynamin-2 and the transport protein particle (TRAPP) III complex [53,54]. How ATG9 can contribute to autophagosome membrane growth without integrating itself is an open question [55]. One possibility is that ATG9 vesicles mediate the recruitment of enzymes that can regulate lipid biogenesis at the phagophore, such as the PI(4)P-kinase, PI4KIII $\beta$  [17].

The specific signals that direct ATG9-containing vesicles to the phagophore remain largely unknown. Phosphorylation of ATG9 by ULK1 can regulate its interaction with the coat proteins, AP-1 and AP-2, stimulating the redistribution of ATG9-containing vesicles from the plasma membrane to the cytosol [56]. However, the localisation of ATG9 to autophagic membranes can occur in the absence of the ULK complex [19,55,57,58]. Interactions between ATG9/Atg9 and ATG2/Atg2 proteins have been reported in mammalian and yeast cells [59,60]. In yeast cells lacking Atg9 or expressing Atg2 mutants unable to bind Atg9, Atg2 could still be recruited to phagophores but exhibited an altered distribution [7,60]. On the other hand, ATG9/Atg9 accumulated at autophagic sites in the absence of ATG2/Atg2, suggesting that ATG2 proteins or autophagosome maturation may be required for the displacement of ATG9 from the phagophore [59,60]. Further studies are required to understand the vesicular trafficking events that recruit and displace ATG9 at autophagic structures.

## PI(3)P-dependent recruitment

### WIPIs

There are four WIPI proteins in mammalian cells, termed WIPI1-4 [61,62]. Characteristic of these proteins is their ability to fold into a seven-bladed  $\beta$ -propeller structure through their WD domains and their affinity for PI(3)P and PI(3,5)P<sub>2</sub> via a two-site recognition motif (FRRG/LRRG) [63-65]. This motif is adjacent to a hydrophobic loop that is hypothesised to insert into membranes and provide an additional mechanism for stable membrane binding [64].

All four WIPIs are recruited to punctate structures upon nutrient starvation and colocalise with a variety of early and late autophagic markers [66]. As expected

based on the presence of a PI(3)P-binding domain, inhibiting PI(3)P synthesis prevents the localisation of WIPI1-3 to the phagophore during mTORC1 inhibition [66,67]. It was also shown that loss of the phosphoinositide binding motif in WIPI2b (an isoform of WIPI2) significantly reduces its recruitment to punctate structures [68], and this is also likely to be the case for WIPI1-3. Interestingly, whilst inhibiting PI(3)P synthesis prevented the localisation of WIPI2 to punctate structures, biochemical fractionation showed that the recruitment of WIPI2 to membrane fractions was not compromised [62]. This indicates that, in fed conditions, WIPI2 may be tethered to membrane compartments via unidentified protein-protein or lipid-protein interactions. Indeed, WIPI2, and to a lesser extent WIPI1, can bind to the recycling endosome-resident Rab GTPase, Rab11A, which contributes to autophagosome formation and cargo recognition in a manner independent of PI(3)P binding [69]. In the case of WIPI4, its ability to form punctate structures is not sensitive to VPS34 inhibition [66]. This may be explained by the affinity of WIPI4 for additional lipid species, such as PI(4)P and PI(5)P, as shown by lipid-protein overlay experiments [66]. The mechanism that allows WIPI4, but not other WIPIs, to recognise additional lipid species and the relevance of its interaction with ATG2 (see below) remains unclear.

The exact functions of the different WIPI proteins and whether they have redundant and/or independent functions remain topics of ongoing research. Genetic knockdown or knockout experiments show that in the absence of either WIPI1 or WIPI2, LC3 lipidation is not fully abolished, or in some cases not affected, although phagophore growth is abrogated [10,62,66,70]. This is intriguing as the recruitment of downstream effectors, such as ATG16L1, is strongly abolished in the absence of WIPI1 or WIPI2 [66,68,70]. On the other hand, individual knockdown of WIPI3 or WIPI4 appears to significantly enhance the levels of lipidated and unlipidated forms of LC3 and ATG16L1 puncta under fed, but not starvation, conditions [66].

In support of the WIPIs having independent functions, overexpression experiments show that the formation of WIPI1 and WIPI2 puncta are significantly enhanced in ATG5 knockout cell lines, whilst WIPI3 and WIPI4 puncta are unaffected [66]. The relevance of the earlier recruitment of WIPI1 and WIPI2 correlates with their abilities to bind ATG16L1, which is not observed for WIPI3 and WIPI4 [66,68]. Since WIPI2 exhibits stronger binding for ATG16L1 compared to WIPI1 [66,68], it is thought to provide one mechanism of bridging the downstream lipidation

machinery to PI(3)P-enriched phagophores. Additional functions of WIPI proteins may exist that facilitate autophagosome biogenesis downstream of LC3 lipidation. One of these functions, for example, may be to mediate the displacement of various PI(3)P effectors over the course of the pathway to enable for the efficient progression of autophagosome biogenesis, as shown by the ability of overexpressed WIPI1 to displace WIPI2 from punctate structures [62].

### ATG5 complex

The ATG16L1-ATG5-ATG12 complex (referred to herein as the ATG5 complex) harbours an E3-like conjugation activity that plays an essential role in the lipidation of ATG8 proteins during autophagy [71]. The ATG5 complex can stimulate LC3 lipidation *in vitro* [10,72] and when ectopically tethered to membranes in cells [73]. Whilst other E1- and E2-like ligases (namely ATG7 and ATG3, respectively) are also essential for ATG8 lipidation, the ATG5 complex is the only one reported to localise on autophagic membranes and understanding its recruitment to these structures has therefore been a longstanding question in the field. The ability of ATG16L1 to form oligomers contributes to the formation of a complex of roughly 800 kDa that acts as a scaffold on the phagophore during its expansion [74,75]. Amongst the ATG5 complex members, ATG16L1 is responsible for tethering the complex to the phagophore, whilst the ATG5-ATG12 conjugate is dispensable for this activity [70,73,74].

It appears that there are several potential mechanisms for recruiting ATG16L1 to the phagophore (Fig. 2B). These most likely act together to ensure the timely regulation of ATG5 complex activity during autophagy. A number of ATG16L1-interacting partners have been described including the autophagic proteins FIP200 and WIPI2 [68,76,77] and PI(3)P [70]. Interestingly, these interactions are adjacent to one another and span amino acids I171/K179/R193 (PI(3)P binding), E226/E230 (WIPI2b binding) and K229 to V242 (FIP200 binding) of mouse ATG16L1. Live imaging analyses show that ATG5 and FIP200 are recruited to autophagic structures simultaneously [78], whilst WIPI2 puncta appear to precede ATG16L1 recruitment [68]. Thus, different interacting partners may act at distinct stages of autophagosome biogenesis or in response to varying autophagy-inducing stimuli.

Direct binding of ATG16L1 to the PI(3)P effector, WIPI2, and to PI(3)P corroborates data highlighting the sensitivity of ATG16L1 puncta to the inhibition of PI(3)P synthesis [19,68,70]. This dual binding may stabilise ATG16L1 on the phagophore and allow for the

asynchronous recruitment of ATG16L1 and WIPI2 [68,70]. On the other hand, binding of ATG16L1 to FIP200 may be important to relay upstream signals to induce autophagy during mTORC1 inhibition [76]. An interaction between ATG16L1 and ubiquitin has also been proposed to be required for autophagy in the absence of FIP200 [79], highlighting an additional mechanism that may be required to efficiently stabilise the ATG5 complex at the phagophore or during cargo recruitment. These various mechanisms of anchoring ATG16L1 at the phagophore may also provide a degree of redundancy and explain why LC3 lipidation can still occur in WIPI2 and FIP200 knockout cells, whilst it is completely disrupted in ATG16L1 knockout cells [10,68,79]. The hierarchy of these binding partners of ATG16L1 and how they can crosstalk to mediate its efficient recruitment to the phagophore remain to be fully determined. The complexity of ATG16L1 membrane recruitment is further underscored by findings that these protein binding partners are dispensable for its role in mediating LC3 lipidation during mTORC1-independent autophagy or on single membranes [76,80].

Surprisingly, persistent localisation of ATG16L1 to membranes and its overexpression in some cell lines did not enhance LC3 lipidation but rather suppressed it [70,73,81]. One potential explanation is that the displacement of the ATG5 complex from membranes is important for autophagy. This inhibitory effect of ATG16L1 was also seen using a mutant within its CCD that harbours increased lipid binding affinity [70] or using a CAAX fusion of an N-terminal fragment of ATG16L1 with an intact CCD [81]. Consistent with these data, expression of the CCD alone in cells can mediate the inhibitory effects of overexpressing ATG16L1 [73]. Transient expression of ATG16L1 may also result in its predominant recruitment to Rab11-positive recycling endosomes [82]. Whilst this ectopic localisation of ATG16L1 can have deleterious effects on autophagy, the presence of ATG16L1 on recycling endosomes and the plasma membrane under more physiological conditions may favour autophagosome biogenesis [51,83]. Thus, it can be speculated that the recruitment of ATG16L1 to membranes must be tightly regulated in order to facilitate the efficient lipidation of LC3. Post-translational modifications of ATG16L1 (such as phosphorylation and methylation) have been recently described; however, these appear to only affect autophagy induced by specific stimuli [84-86].

Of interest, sequences within ATG16L1 were also shown to mediate its targeting to single membrane compartments, where it can mediate the noncanonical



lipidation of ATG8 proteins [10,80]. Residues within the C-terminal half of ATG16L1 that lie within the  $\beta$ -isoform-specific region (VRV motif) and the seven WD40 domains (including amino acids F467 and K490 of mammalian ATG16L1) are required for this by mediating its interaction with lipids and an unknown factor, respectively. The existence of this additional membrane targeting mechanism and the identification of factors that determine whether ATG16L1 localises to the phagophore or to single membranes are likely to aid our understanding of how this crucial protein is activated and specifically targeted to autophagic membranes.

## ATG2

ATG2 proteins (ATG2A and ATG2B in mammals) are large and functionally redundant proteins with essential roles in autophagy (Fig. 2C) [87]. The co-depletion of ATG2A and ATG2B in mammalian cells resulted in the accumulation of lipidated LC3 and the autophagic receptor p62, highlighting their roles in later stages of autophagosome biogenesis, including phagophore elongation and closure [59,87,88]. These functions of ATG2 proteins may be attributed to their membrane tethering activities (via protein and lipid interactions), lipid transfer activities (via an N-terminal domain homologous to Chorein/VPS13) and interactions with the ATG8 family of proteins [88–94]. Furthermore, ATG2 can localise to both lipid droplets and autophagic membranes and can control membrane dynamics during autophagosome assembly [87]. The presence of a highly conserved cysteine–alanine–aspartic acid sequence (known as the CAD domain) within the middle region of ATG2 has been postulated to mediate binding to lipids and contribute to its localisation at the ER, although the latter has not been directly tested [90]. This localisation coupled with its lipid transfer activity may provide a mechanism for lipid supply during autophagosome growth.

PI(3)P is required for the recruitment of ATG2 to the phagophore but not to lipid droplets in mammalian cells [87]. In addition, deleting sequences within the N-terminal region of ATG2 does not disrupt its localisation to lipid droplets but abrogates its colocalisation with WIPI2, suggesting that the ability of ATG2 to localise to lipid droplets and the phagophore requires distinct mechanisms [95]. The PI(3)P-dependent recruitment of ATG2 was initially thought to require the formation of a stable complex between ATG2 and the PI(3)P effector, WIPI4. This was originally described in yeast where the Atg2-interacting partner, Atg18 and its PI(3)P binding site were shown

to be required for the recruitment of Atg2 to the phagophore [7,96]. However, more recent data from yeast and mammalian studies show opposing results in that Atg18/WIPI4 binding appears to be rather dispensable for Atg2/ATG2 recruitment to the phagophore and autophagosome closure [59,88,97]. In addition, the autophagy defect detected upon WIPI4 genetic inhibition does not resemble that of ATG2A/B inhibition [66,87]. These findings suggest that the functional relevance of the ATG2–WIPI4 interaction and the mechanism underlying the PI(3)P-dependent recruitment of ATG2 have not yet been fully uncovered.

Whilst studies of mammalian ATG2 have only emerged in the past few years, yeast Atg2 has been more extensively studied. Low homology in amino acid sequences between yeast and mammalian ATG2 proteins (13%) may suggest evolutionary diversions in the molecular mechanisms that underlie the roles of ATG2 during autophagy [88]. In support of this, it has been shown that the expression of human ATG2A in an *atg2 $\Delta$*  yeast strain does not rescue the autophagic defect in yeast cells, although ATG2A can still localise to the phagophore [98]. Furthermore, yeast Atg2 can directly bind to PI(3)P, whereas purified mammalian ATG2B can bind to liposome preparations independently of this lipid [90,99]. On the other hand, a direct interaction between Atg2 and Atg9 has been reported [60], which appears to be conserved in mammals [59]. As mentioned above, the ability of Atg2 to interact with Atg9 is required for its proper localisation to the phagophore. In the absence of Atg9 binding, the localisation of Atg2 is no longer confined to ER–phagophore contact sites, impairing autophagic flux in yeast cells [60]. How the binding of Atg2 to Atg18 and lipids contributes to the proper maturation of autophagosomes in this model needs to be further elucidated.

The recruitment of ATG2 proteins to the phagophore in mammalian cells appears to be dependent on a number of interactions. Truncation analyses of ATG2A suggest that deleting an amphipathic helix near its C-terminal end completely abolishes its punctate localisation to both autophagic structures and lipid droplets [87]. Interestingly, sequences within this region of ATG2 were found to mediate its binding to mitochondrial membrane proteins, translocase of the outer mitochondrial membrane (TOM) 40 and TOM70, at the mitochondrial-associated ER membrane (MAM) and were termed the MAM–localisation domain (MLD) [59]. MLD mutants of ATG2, which are unable to interact with the mitochondrial proteins, harboured a diffused cytosolic localisation and were deficient in reconstituting autophagic flux [59]. Similar to findings in yeast, the ability of mammalian ATG2

to interact with ATG9 has been reported [59]. Cells lacking ATG2 accumulated ATG9 at the phagophore, suggesting that autophagosome maturation or a direct ATG2–ATG9 interaction is required for the recycling of ATG9 vesicles from the phagophore [59]. ATG9, on the other hand, was shown to be required for the interaction of ATG2B with a component of the TRAPP complex, TRAPPC11, which is involved in membrane trafficking. The formation of this complex is required for ATG2B–WIP14 recruitment to the phagophore and autophagic flux, suggesting that vesicle trafficking events may be required for the efficient localisation of ATG2 proteins to the phagophore [100].

### ATG8 proteins and cargo receptors

The mammalian ATG8 family is composed of six members: LC3A, LC3B, LC3C, GABARAPL, GABARAPL1 and GABARAPL2. Through pulse-chase and mutagenesis experiments, it was shown that these proteins are initially synthesised in a pro-form, rapidly processed to a cytosolic form (known as the ‘-I’ form) and later converted into a membrane-bound form upon autophagy induction (known as ‘-II’ form) [101,102]. This membrane-bound form represents the direct conjugation of ATG8 to the headgroup of phosphatidylethanolamine (PE) and requires the activities of ATG7, ATG3 and the ATG5 complex [10,71,103]. As mentioned above, membrane recruitment of the ATG5 complex can initiate ATG8 lipidation and is therefore tightly regulated. ATG3 can directly interact with ATG12 and subsequently catalyse the lipid conjugation of LC3 when in close proximity to membranes [104,105]. Interestingly, ATG3 harbours an amphipathic helix that exhibits a preference for highly curved membranes, such as those found on the leading edge of the phagophore, possibly indicating that the ATG5 complex may preferentially recruit ATG3 to these sites [9].

The processing of ATG8s to the ‘-I’ form, known as priming, is achieved through the activity of four isoforms of the ATG4 protease (ATG4A–D), but preferentially via ATG4B based on kinetic analyses [106–109]. The delipidation of ATG8s from their ‘-II’ form to the ‘-I’ form also requires ATG4 proteins, and the kinetics of this are dependent on the nature of the ATG8 but not the curvature of the membrane onto which that ATG8 is lipidated [108]. This activity of ATG4 is required to recycle ATG8 back to the cytosol and can be regulated by both the phosphorylation of ATG8 family members [110] and by post-translational modifications on ATG4 [111–113].

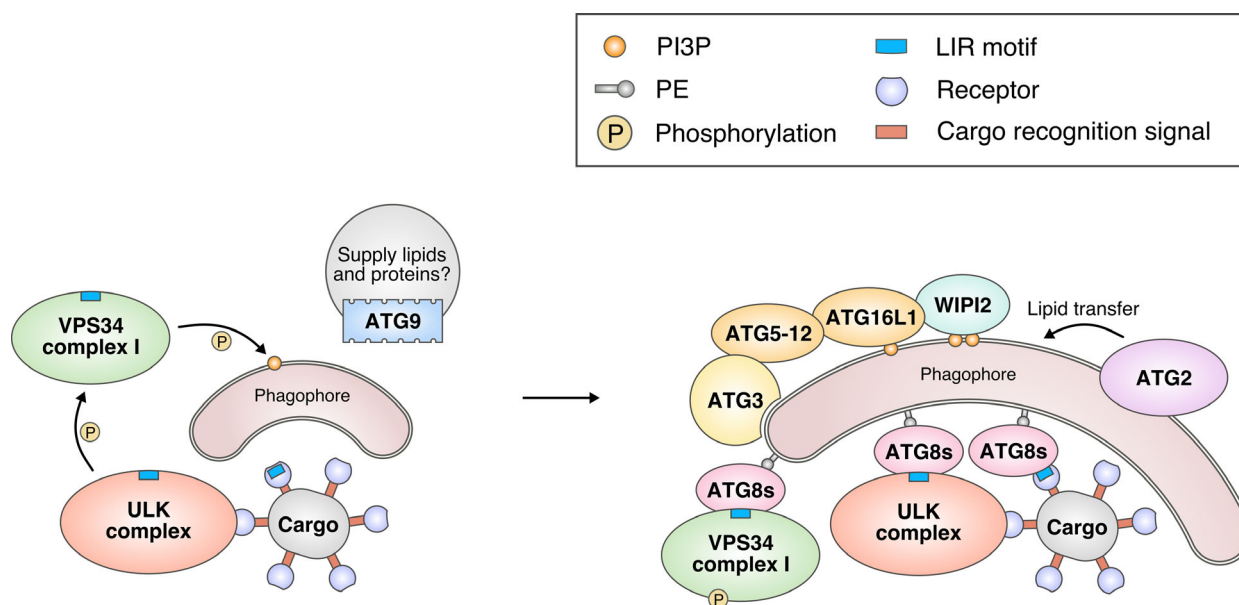
There are two main accepted functions for the ATG8 proteins: promoting phagophore maturation

[114–116] and recruiting specific cargo to the phagophore [117]. The identification and characterisation of an LC3-interacting region (LIR) was initially described in cargo receptor proteins, such as p62, containing the amino acid sequence: [W/F/Y]-X-X-[L/I/V], and is thought to aid in anchoring cargo receptors to the phagophore [117–120]. The existence of LIRs in further upstream autophagy players, such as the ULK and VPS34 complexes, indicates a potential role of ATG8s in scaffolding the assembly of the early autophagy machinery and/or a downstream function of these upstream players during autophagosome maturation or lysosome fusion (Fig. 3) [121,122].

Recent studies have started to reveal mechanisms by which the autophagic cargo receptor proteins can be recruited to membranes in the absence of ATG8 binding. The accumulation of p62 at autophagic structures and the ER can occur in ATG3 or ATG5 knockout cell lines, indicating that binding to lipidated ATG8s is dispensable for this activity [123]. Similarly, NBR1, another LC3-interacting cargo receptor protein, was found to be recruited to autophagic structures in the absence of PI(3)P synthesis and independently of p62 [123]. In addition, a number of interactions have been described between cargo receptors and upstream autophagy players, including Alf1/ATG5 and p62/FIP200 during aggrephagy [34,124], TECPR1/ATG5/WIP12 during xenophagy [125] and CCPG1/FIP200 during ER-phagy [126], to name a few. The selectivity of autophagic cargo receptors and their encapsulation by the autophagosome are topics of extensive research and have been reviewed elsewhere [117].

### Factors affecting ATG protein recruitment

The recruitment of ATG proteins to the autophagosome membrane occurs in a dynamic and reversible manner. Understanding the underlying mechanisms could provide robust means to regulate autophagy in experimental settings and in disease. Potential signals, such as post-translational modifications, that may govern direct membrane binding and/or protein–protein interactions remain to be elucidated. Such signals are likely to be transient and reversible events and may require specific biochemical approaches to be identified. In the case of ATG7 and ATG3, post-translational modifications, such as acetylation and oxidation, have been identified and shown to modulate autophagy in response to nutrient starvation and ageing [127,128]. Additionally, changes in the intracellular microenvironment, including calcium availability and pH, have been shown to regulate autophagosome



**Fig. 3.** Cargo-dependent autophagosome biogenesis. During selective autophagy, the cargo destined for degradation and their receptors are thought to recruit the autophagic machinery. In this example, binding between the ULK complex and a cargo receptor, such as p62, leads to phagophore biogenesis around the cargo through the activities of the VPS34 complex I and ATG9. This results in the recruitment of downstream proteins, such as WIPI2, ATG2 and the ATG5 complex, ultimately leading to ATG8 lipidation and phagophore growth. The presence of LIR motifs in cargo receptors and certain ATG proteins (including the ULK and VPS34 complexes) results in their proximity to ATG8s and autophagic structures. Upon maturation, autophagosomes can fuse with lysosomes leading to the degradation of their contents (not shown).

biogenesis [129,130]. The mechanisms through which the latter occurs is largely underexplored but may directly regulate the membrane recruitment of ATG proteins.

In addition to understanding the mechanisms that mediate the recruitment of ATG proteins to autophagosome membranes, the displacement of many ATG complexes from the phagophore remains an open question. Most ATG proteins are displaced from the outer membrane of the autophagosome prior to or following their maturation, whilst ATG8s lipidated to the inner membrane remain bound [78,131]. Inhibiting autophagosome maturation by disrupting ATG8 lipidation, for example in the absence of ATG3 or ATG7, accumulates upstream complexes, such as the ATG5 complex [76,132]. This indicates that the maturation of autophagosomes, and potentially ATG8 lipidation, may act in an unknown mechanism to displace many of the upstream machinery. In addition, hydrolysis of PI(3)P by lipid phosphatases, including a family of myotubularin-related phosphatases, may be an additional mechanism to allow the displacement of PI(3)P-binding proteins from autophagic membranes [133–135].

## Conclusions and Final remarks

In summary, the specific hierarchy and regulation of ATG protein membrane recruitment during autophagosome biogenesis merits further study. Given the dynamic nature of autophagosome formation, it is likely that a combination of genetic and biochemical approaches will be required in order to fully dissect how various autophagy players are activated, recruited to the phagophore and eventually recycled back to the cytosol. Furthermore, this may be complicated by the apparent existence of compensatory mechanisms in cells, both with respect to the tethering of proteins to membranes and to the biogenesis of lipid species. Molecular insights from understanding how some ATG proteins can facilitate ATG8 lipidation on single membrane compartments could aid in dissecting their regulation at the autophagosome, although this may require distinct mechanisms and ATG complexes [80].

At the moment, understanding membrane recruitment of ATG proteins mainly relies on fluorescent imaging of endogenously or exogenously expressed proteins with ‘puncta’ formation as a readout [136]. These assays have various limitations including the

lack of specific antibodies to detect some endogenous proteins, the ectopic localisation of overexpressed constructs, the lack of tools to visualise endogenous autophagosome initiation sites (such as omegasomes and ER contact sites), and the inability to distinguish defects in the initial recruitment of ATG proteins to the phagophore versus phagophore growth. Localisation of ATG proteins may also depend on the stimulus used to trigger autophagy, cell type and fixation methods. In addition, it is possible that cells adapt to prolonged gene knockout and that reconstitution experiments may not reflect events occurring under physiological conditions. The development of additional tools and biochemical techniques to specifically isolate autophagic structures from cells would aid our understanding of how ATG proteins are regulated during autophagy.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

LJD, ANM and NG drafted, edited and revised the manuscript. LJD and NG designed the figures.

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